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(54) **Regulatory region cloning and analysis plasmid for bacillus.**

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(73) Proprietor: **GIST-BROCADES N.V.**
Wateringseweg 1
NL-2611 XT Delft (NL)

(72) Inventor: **van EE, Jan Hendrik**
Roekendaal 2
NL-2914 EZ Nieuwerkerk a/d ijssel (NL)

(74) Representative: **Huygens, Arthur Victor, Dr. et al**
c/o Gist-Brocades N.V. Patent & Trademarks Department Wateringseweg 1 PO Box 1
NL-2600 MA Delft (NL)

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Description

Field of the Invention

Plasmids are provided for isolating and evaluating regulatory regions of genes in Gram-positive bacteria, particularly *Bacillus*. Synthetic regulatory regions are provided associated with convenient restriction sites, whereby various cassettes can be prepared involving at least one of a promoter sequence, a ribosomal binding site sequence, and a signal sequence functional in *Bacillus,* where one or more of these regions may be substituted by the region to be evaluated. A plurality of restriction sites are provided for ease of substitution of one of the regions with a different region.

Background of the Invention

In the use of genetic manipulation techniques in microorganisms, the genus *Bacillus* has, after *E. coli,* in recent years also formed the subject of extensive investigation. See, for example, *Dubnau,* in Experimental Manipulation of Gene Expression, Academic Press, (1983) 33—51 and *Doi,* Biotechnology and Genetic Engineering (1984) *2*:126—155. *Bacilli* have now been used for a long time in the fermentation industry. *Bacilli* offer numerous advantages, such as good growth on inexpensive base materials, and in contrast to *E. coli,* do not produce any endotoxins. Furthermore, *Bacilli* are capable of secreting proteins into the growth medium, in particular certain types of enzymes such as proteases and amylases, frequently produced in large amounts by *Bacilli*. These enzymes may be relatively inexpensively and conveniently isolated from the fermentation medium.

Because of the attractiveness of *Bacilli* as a host for the production of homologous or heterologous peptides, it is substantial commercial interest to be able to make use of particular sequences associated with transcriptional and translational regulation, which would allow for efficient expression and secretion of the peptides of interest. There is, therefore, substantial interest in ways for isolating and analyzing these sequences from *Bacillus* or other sources, which would allow for the efficient screening of the sequence.

Description of The Relevant Literature

Plasmids for *S. aureus* are capable of extrachromosomal maintenance in *B. subtilis (Ehrlich,* Proc. Natl. Acad. Sci. USA (1977) *74*: 1680—1682). Various prokaryotic and eukaryotic heterologous proteins have been cloned in *B. subtilis,* usually at low expression levels. See, for example, *Kovacevic et al.,* J. Bacteriol. (1985) *162*:521—528; *Saunders et al., ibid.* (1984) *157*:718—726; *Ohmura et al.,* Third International Conference on Genetics and Biotechnology of Bacilli, Stanford, USA (1984); *Lundström,* FEMS Letters (1984) *23*:65—70, *Palva et al.,* Gene (1983) *22*:229—235; *Lundström et al.,* Virus Res. (1985) *2*:69—83; *Hardy et al.,* Nature (1981) *293*:481—483; *Mosbach et al.,* Nature (1983) *302*:543—545; *Chang et al.,* NSC Ser. (1982) *4*:254—261; *Williams et al.,* Gene (1981) *16*:199—206 and *Flock et al.,* Mol. Gen. Genet. (1984) *195*:246—251.

In order to arrive at an economically acceptable expression level, it is necessary to achieve improvements in the cloning system. In this context consideration can in particular be given to a modification of the promoter, in order to improve the transcription efficiency of the heterologous gene, a modification of the ribosomal binding site (Shine-Dalgarno), in order to improve the translation efficiency, and/or a modification of the signal sequence, in order to improve the secretion of the desired heterologous protein product.

Various proposals have been made for combining synthetic or naturally occurring promoters with a gene other than the wild-type gene. See for example *Williams et al.,* J. Bacteriol. (1981) *146:*1162—1165; *Schoner et al.,* Gene (1983) *22*:47—57, who describe plasmid pPL 603; *Goldfarb et al.,* Nature (1981) *293*:309—311 (plasmid pGR 71); *Band et al.,* Gene (1983) *26:*313—315 (plasmid pCPP 3—4); and *Donelly and Sonnenshein,* J. Bacteriol. (1984) *157:*965—967 (plasmid pCED 6).

However, these plasmids have the disadvantage that they are fairly large and comprise only 1 or 2 promoter insertion sites. Moreover, they are almost all based on chloramphenicol acetyl-transferase as the indicator enzyme, and comprise an inducible Shine-Dalgarno sequence (pPL 603) or an inactive Shine-Dalgarno sequence (pGR 71). Consequently, promoters can be isolated only if a fusion protein is formed with the chloramphenicol acetyl-transferase, that is to say if a *Bacillus* Shine-Dalgarno sequence is also co-cloned and the reading frame is in phase with that of the chloramphenicol acetyl-transferase.

For discussion of the Shine-Dalgarno sequence and the initiation codon, see *Hui et al.,* EMBO J. (1984) *3*:623—629; *De Boer et al.,* DNA (1983) *2*:231—235; *Band and Henner,* Biochem. Soc. Symp. (1984) *48*:233—245; and EP—A—116411.

Concerns involved with evaluation of regulatory sequences have been expressed by *Hall et al.,* Nature (1982) *295*:616—618; *Shpaer,* Nuclei Acids Res. (1983) *13*:275—289 and *Tessier et al., ibid.* (1984) *12*:7663—7675.

Stanssens et al., Gene (1985) *36*:211—213 describe the effect of alterations of the sequence upstr am from th Shine-Dalgarno region; *Iwakura et al.,* J. Biochem. (1983) *93*:927—930 describe the construction f plasmid vectors employing the *dhfr* gene; and *Hosoya et al.,* Agri ultural and Biological Chemistry (1984) *48*:3145—3146, describe the construction of a promoter cloning vector in *P. aeruginosa.*

Ohmura et al., J. Bi chem. (1984) *95*:87—93, describe a *B. subtilis* secretion vector system employing th alpha-amylase promoter and signal sequence region; *Enger-Valk et al., Gene (1981) 15*:297—305

describe a vector for cloning f promoters; *Tsoi et al.,* Gen tika (Mosc w) (1981) *17*:2100—2104 describe the cloning and expr ssion of pr m ter fragm nts of *B. thuringiensis* DNA in *E. coli* cells; *Moran et al.,* M l. Gen. Genet. (1982) *186*:339—346 describ nucleotide sequences that signal th initiation of transcripti n and translation in *B. subtilis;* see also EP—A—134048.

Summary of the Invention

Novel DNA sequences and combinations of sequences are provided for the isolation of DNA fragments that can function as promoter in *Bacilli.* Said sequences are characterized by a structural gene, including a functional siognal sequence, and a synthetic ribosomal binding site. The gene product encoded by the structural gene is, with the aid of the signal sequence, secreted by the host organism. Its activity can be easily assayed for, provided that upstream of the ribosomal binding site a DNA fragment is inserted, preferably using one of the available unique restriction sites. Said sequences are further characterized in that regulatory regions concerned with transcription, translation and secretion, and the structural gene itself can be conveniently exchanged by the presence of unique restriction sites bordering these regions. pPROM 54 is a promoterless plasmid useful in the screening of fragments for the presence of promoters in proper orientation and spacing.

Brief Description of the Drawings

Fig. 1 depicts the nucleotide sequence of the promoter region, Shine-Dalgarno sequence, and signal sequence of *B. licheniformis* alpha-amylase with the predicted amino acid sequence of the signal sequence;

Fig. 2 depicts in diagrammatic form the removal of the natural alpha-amylase Shine-Dalgarno sequence and promoter sequence and the integration of the synthetic Shine-Dalgarno sequence in M13mp10;

Fig. 3 depicts the plasmid pPROM 54;

Fig. 4 depicts the plasmid pPROM 55s; and

Fig. 5 depicts the plasmid pPROM 37s.

Description of The Specific Embodiments

Novel DNA sequences are provided in which a regulatory domain is provided. The domain allows for insertion and exchange of individual functional elements of the domain, as well as subunits of a functional element. The domains provide for transcriptional and translational regulatory elements, including elements affecting such regulation, such as operators, enhancers, activators, or the like. Of particular interest is a domain which provides for regulation of transcription, regulation of translation, both a ribosomal binding site and an initiation codon, a signal sequence for secretion and, as appropriate, a structural gene in reading phase with the signal sequence, which signal sequence may or may not include a processing signal for peptide cleavage.

Thus, in accordance with the present invention there is provided a plasmid capable of replication in *Bacillus* useful in evaluating regulatory or signal sequences for expression of a hybrid gene having a domain comprising at least one restriction site and as elements for substitution: (1) as a promoter region, the *B. licheniformis* promoter sequence in pPROM 3—4C, which plasmid has the deposit accession number CBS 699.85, the bacteriophage promoter of pPROM SPO2, which plasmid has the deposit accession number CBS 698.85, or the −10 and −35 sequences of a synthetic promoter region, (2) a ribosomal binding site, with the proviso that when said promoter region is absent, said ribosomal binding site is synthetic, and (3) a signal sequence functional in *Bacillus;* with unique restriction sites between the −10 and −35 consensus sequences of any synthetic promoter region, between the promoter region and the ribosomal binding site, proximal to the initiation codon and at the 3'-terminus of the signal sequence, wherein at least two of said elements are not naturally linked, with the proviso that one of said elements may be replaced by a unique restriction site, and in that event said signal sequence is the alpha-amylase signal sequence and is joined to a structural gene in reading phase encoding mature alpha-amylase.

The domain comprises at least one of the following sequences:

A) 5'-GATCCAAGGAGGTGAT-3'

B) 5'-CTAGATCACCTCCTTG-3'

C) 5'-AATTCTTGACAAAGCTTC-3'

D) 5'-TCGAGAAGCTTTGTCAAG-3'

E) 5'-TCGAGACTGATATAATGAGCT-3'

F) 5'-CATTATATCAGTC-3'

G) 5'-AATTCAGGATTTATGAAGCTTC-3'

H) 5'-TCGAGAAGCTTCATAAATCCTG-3'

I) 5'-TCGAGGGAATTGTTTGAGCT-3'

J) 5'-CAAACAATTCCC-3'

According to an aspect of the invention the promoter is replaced with a sequence which includes at least three unique restriction sites.

The constructs involve selected promoter sequences which bind to the *Bacillus* σ^{55}, σ^{37}, σ^{32}, σ^{29}, σ^{28} or other RNA polymerases, provided with convenient restriction sites for introduction into and excision from a vector. Also provided is a Shine-Dalgarno sequence with a plurality of convenient restriction sites so as to allow for introduction to and excision from a vector. A signal sequence for secretion is provided with convenient restriction sites for insertion and excision of the signal sequence, as well as insertion downstream and in reading frame with the signal sequence of a structural gene of interest.

A convenient *Bacillus* replication system is employed, such as the replication system pUB110. The vectors which are available will normally have a marker for selection, which marker allows for resistance to a cytotoxic agent such as an antibiotic, *e.g.* kanamycin, chloramphenicol, tetracycline, streptomycin, etc.; heavy metal, or the like; or for complementation in an auxotrophic host. One or more markers may be present, particularly where a shuttle vector is employed, where the vector is capable of replication in two or more hosts. Conveniently, the vector may include a replication system for replication in two or more hosts. Conveniently, the vector may include a replication system for replication in *E. coli*, so as to allow for cloning and extension of the DNA after each of the manipulative steps involved with the formation of the construct.

Suitable plasmids according to the invention include pPROM 54, pPROM 55s, pPROM SPO2 and pPROM 3—4C, which have the deposit accession numbers CBS 696.85—699.85, respectively. In these plasmids, at least one but not all of the elements may be substituted by a different sequence having the same function.

In a further aspect the invention provides a *Bacillus* host comprising a plasmid as defined hereinbefore.

Suitable host microorganisms of the *Bacillus* species are *B. subtilis, B. licheniformis, B. amyloliquefaciens* and *B. stearothermophilus*.

At least one and preferably two of the subject sequences providing for a particular regulatory region or signal sequence are employed in combination with the sequence to be evaluated.

The first sequence to be considered is the promoter sequence. This region is involved with the binding of the σ^X-RNA polymerase wherein X intends any of the RNA polymerases indicated previously. Th sequence may be synthetic or wild-type. One synthetic promoter, will have the following sequence:

AATTCTTGACAAAGCTTCTCGAGACTGATATAATGAGCT

GAACTGTTTCGAAGAGCTCTGACTATATTAC

The subject synthetic sequence which is recognized by the σ^{55}-RNA polymerase has a number of significant features, allowing for the individual substitution of the −10 region or the −35 region by digestion with restriction enzymes *Hind*III or *Xho*I and *Sst*I or digestion with *Eco*RI and *Hind*III or *Xho*I, respectively. The entire synthetic promoter may be substituted by digestion with *Eco*RI and *Sst*I.

A second synthetic promoter for the σ^{37}-RNA polymerase will have the following sequence:

AATTCAGGATTTATGAAGCTTCTCGAGGGAATTGTTTGAGCT

GTCCTAAATACTTCGAAGAGCTCCCTTAACAAAC

This sequence enjoys similar benefits as described for the σ^{55}-RNA polymerase promoter.

Other promoters which may be used to advantage include wild-type promoters, such as the wild-type promoter found in plasmid pPROM 3—4C, deposited at th CBS on Nov mber 5, 1985, under No. 699.85, where the prom ter is a chrom somal promoter sequence derived from *B. licheniformis*. The above plasmid is derived from the plasmid pPROM 54, where th insertion of the *B. licheniformis* prom ter results in an increase in alpha-amylase production in *B. subtilis* of 35%, c mpared with the plasmid pGB 33 carrying the original promoter and Shine-Dalgarno sequences.

Another promoter derived from a bacteri phage promoter sequence is present in plasmid pPROM

SP02, deposited at the CBS on November 5, 1985, under No. 698.85. This plasmid is also derived from the plasmid pPROM 54, by insertion of a promoter sequence from the bacteriophag sequence derived from the plasmid pPL 608, which is described by *Williams et al.* (1981), *supra.* The insertion of the bacteriophage promoter results in an increase of alpha-amylase production in *B. subtilis* of 37% over the natural promoter.

These various promoter sequences may be substituted by any other promoter sequence, from any source, where it is intended to determine the efficiency of such promoter in a *Bacillus* host. Thus, promoter sequences, or portions of promoter sequences, either synthetic or natural, such as the −10 region or the −35 region consensus sequences may be incorporated for evaluation, by ligating such DNA sequences to one or more of the other sequences provided in accordance with this invention.

The next region of interest is the Shine-Dalgarno sequence or ribosome binding region. For this purpose, a synthetic region may be suitably employed having the following sequence:

GATCCAAGGAGGTGAT

GTTCCTCCACTAGATC

The third sequence which is employed in the subject invention is the signal sequence of alpha-amylase, which may be conveniently joined to the region coding for the mature alpha-amylase or to a different gene resulting in a hybrid gene. A convenient restriction site is provided between the alpha-amylase signal sequence and the remainder of the alpha-amylase gene, so as to allow for substitution of the region coding for the mature alpha-amylase.

Each of the fragments which are employed provide for one or more restriction sites which allow for introduction and excision of the individual fragments. Thus, polylinkers or one or more restriction sites, desirably unique restriction sites, are present within and between the regions for convenient insertion or excision of sequences. Usually, the polylinker will have at least two restriction sites and usually not more than about six restriction sites, more usually not more than about four restriction sites, frequently unique restriction sites. Exemplary restriction enzyme recognition sites have been indicated previously.

The constructs of the subject invention may be prepared in accordance with conventional ways. The substitution by other sequences of the above regions may require modification of such other sequences. Modifications may include the use of linkers, adapters, *in vitro* mutagenesis, resection, repair, primer repair, or the like, where restriction sites may be introduced or removed, termini modified, etc. After each manipulation, it will usually be desirable to clone the new construct in a convenient host, such as *E. coli,* isolate the new construct and establish the presence of the correct sequence by restriction mapping, sequencing, or the like. Once the construct is completed, it may then be transferred to a vector capable of replication in a *Bacillus* host or a shuttle vector may be used for the construct, which allows for cloning in *E. coli* and direct transfer to the *Bacillus* host.

In still a further aspect of the invention a method is provided for determining the efficiency of one or more functional elements in the production of a peptide in a *Bacillus* host, said method comprising:

growing said host in an appropriate nutrient medium, said host comprising a plasmid capable of replication in *Bacillus* having a domain comprising as elements for substitution: (1) as a promoter region, the *B. licheniformis* promoter sequence in pPROM 3—4C, which plasmid has the deposit accession number CBS 699.85, the bacteriophage promoter of pPROM SP02, which plasmid has the deposit accession number CBS 698.85, or the −10 and −35 sequences of a synthetic promoter region; (2) a ribosomal binding site, and (3) a signal sequence functional in *Bacillus;* with unique restriction sites between the −10 and −35 consensus sequences of the synthetic promoter region, between the promoter region and at the ribosomal binding site, proximal to the initiation codon and the 3'-terminus of the signal sequence, wherein at least one but not all of said elements are substituted by said functional element(s) and wherein a structural gen is in reading phase with said signal sequence; and

determining the amount of said peptide produced in comparison with the amount of peptide produced under comparable conditions with said domain prior to any substitution with said functional elements.

The invention provides further a method for detecting the presence of a sequence capable of initiating transcription in *Bacillus,* said method comprising:

fragmenting DNA from a host having transcriptional initiation regions to less than about 5kbp to produce DNA fragments;

inserting said DNA fragments into a plasmid capable of replication in *Bacillus* and having means for selection of transformants at a restriction site region having at least one unique restriction site and being the upstream region of a screening region, said screening region comprising in the direction of transcription, said restriction site region, a ribosomal binding site region, and a structural gene having a signal sequence and capable of secretion in *Bacillus* to produce a product which is readily detectable by a chemical reaction;

transforming *Bacillus* cells with said plasmid to produce transformants and selecting for transformants by means of said selection means;

screening transformants for secretion of said product by means of said chemical reaction to determin

the presence f a fragment at said restriction site having transcriptional initiation capability.

Of particular interest is the construction of a "fishing" plasmid for screening fragments of DNA f r promoter regions functional in *Bacillus*. The fishing plasmid has two regions necessary for detection of a promoter region. The first region is a promoter screening region and the second region is a transformant selection region. The screening region comprises in the direction of transcription, a region of from about 4 to 100bp having one or more unique restriction sites, usually not more than about 6, and lacking any transcriptional initiation activity. Downstream from the restriction site region is a ribosomal binding site region of from about 5 to 50 bp, including the non-coding nucleotides on either side of the Shine-Dalgarno consensus sequence. The sequence may be natural occurring or synthetic. The ribosomal binding region is followed by a structural gene having a signal sequence for secretion. The structural gene expresses a product which can be readily detected by a simple chemical reaction without the possibility of significant interference from endogenous host materials. Of particular interest is alpha-amylase, which can be detected with a combination of amylose and iodine, where discharge of the color indicates the expression of alpha-amylase and the presence of a promoter in the restriction site region.

The second region provides for selection of transformants. This region will normally encode a gen imparting antibiotic resistance, so that only transformants having the plasmid will survive when grown in medium containing an otherwise cytotoxic amount of the antibiotic. Resistance to neomycin, tetracycline, penicillin, kanamycin, etc. may be provided with the appropriate genes.

Both the screening and selection regions will be joined to a replication system functional in *Bacillus*. Other functional regions may be present in the plasmid, such as a replication system for *E. coli* for cloning.

The fishing or screening method will involve fragmenting DNA from a *Bacillus* or other host e.g. virus, which may have regions capable of transcriptional initiation in *Bacillus*. The fragments may be mechanically produced or by using one or more restriction enzymes, particularly ones that have complementary ends to the restriction sites present in the restriction site region. Fragments as small as 20bp and up to about 5kbp, usually 2kbp, may be obtained for screening, usually from about 50bp to 1kbp. The fragments are inserted into the fishing plasmid in accordance with conventional ways. The resulting plasmid library may then be transformed into a *Bacillus* host and the transformants selected by means of the antibiotic resistance.

Surviving *Bacillus* transformants may then be screened for active promoters by contacting clones with amylose and iodine and isolating those clones which become surrounded by a clear zone.

Transformation of *Bacillus* may be carried out in accordance with conventional ways. See, for exampl *Anagnostopoulos and Spizizen,* J. Bacteriol. (1961) *81*:741—746. Transformants may then be selected in accordance with the nature of the marker.

By employing the subject constructs, structural genes may be evaluated for their ability to be expressed and secreted, where regulatory regions and the structural genes may be mixed and matched to provide for efficient production of the desired product. The production including secretion of a peptide may be determined and compared to other regulatory and functional sequences.

The following examples are offered by way of illustration and not by way of limitation.

Example I

Isolation of Chromosomal DNA

Chromosomal DNA of *B. licheniformis,* strain T5, deposited at the CBS on July 6, 1983, under No. 470.83 (see EP—A—134048) was isolated from 3 l of cultures which had grown overnight at 37°C, und r aeration. The cells were centrifuged for 10 min. in a Sorvall GSA rotor at 10,000 rpm, suspended in 10 ml of sucrose-Tris buffer which contained 25% by weight of sucrose and 50mM Tris-HCl at pH 8.0, and lysed by addition of 0.5 ml of lysozyme solution (20 mg/ml) and subsequently 15 min. incubation at 37°C. After addition of 2 ml of EDTA (0.5 M) and 5 min. incubation at 0°C, 1 ml of 20% by weight sodium dodecylsulfate (SDS) was added. Thereafter, the suspension was extracted with a 1:1 mixture of phenol and chloroform. The supernatant water layer was removed and carefully overlayered with 2 volume units of ethanol, after which the DNA could be isolated with the aid of a glass rod. After dissolution in distilled water to which 10 mg/ml ribonuclease had been added, the mixture was extracted with 1:1 phenol-chloroform, and the product precipitated with 2 parts of ethanol and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0 and 1mM EDTA).

Example II

Isolation of Plasmid DNA

B. subtilis 1—85, containing plasmid pGB 33, deposited at the CBS under No. 466.83 (see EP—A—134048), was cultured overnight in 1 l of minimal medium to which 10 mg/ml neomycin had been added. After centrifuging for 15 min. in a Sorvall model GSA rotor at 5,000 rpm and resuspending in 15 ml of sucrose-Tris, the cells were lysed and treated with EDTA and SDS (see Example I). After addition of NaCl to a final concentrati n of 1M, the lysate was stored overnight at 4°C and subsequently centrifuged for 15 min. at 12,500 rpm in a Sorvall type SS 34 rotor. The uppermost 70% (by volume) of the supernatant liquid was treated for 30 min. at 37°C with 20 µg/ml of DNAse-free RNAse, and extracted with a 1:1 mixture of phenol-chloroform and subsequently with pure chloroform. The DNA was precipitated from the extracted supernatant liquid by addition of 0.2 part of 5M NaCl and 0.25 part of 40% by weight polyethylene glycol

6000, followed by incubation at 4°C overnight. After precipitation and centrifugati n (30 min. at 12,500 rpm, Sorvall type SS 34) the DNA was resuspended in 2—3 ml f TE buffer (see Example I) and kept at pH 12.0 f r 10—15 min. with the aid of 4N NaOH. Thereafter the pH was returned to 8.5 and the mixture was extracted with phenol. After precipitation with ethanol, the plasmid DNA was resuspended in a small volume of TE buffer.

Example III

Oligonuleotide Synthesis

The following oligonucleotide sequences were synthesized with the aid of a Biosearch Synthesis Automation Machine and were purified by means of HPLC, extraction with phenol-chloroform (1:1) and precipitation with ethanol:

A) 5'-GATCCAAGGAGGTGAT-3'

B) 5'-CTAGATCACCTCCTTG-3'

C) 5'-AATTCTTGACAAAGCTTC-3'

D) 5'-TCGAGAAGCTTTGTCAAG-3'

E) 5'-TCGAGACTGATATAATGAGCT-3'

F) 5'-CATTATATCAGTC-3'

G) 5'-AATTCAGGATTTATGAAGCTTC-3'

H) 5'-TCGAGAAGCTTCATAAATCCTG-3'

I) 5'-TCGAGGGAATTGTTTGAGCT-3'

J) 5'-CAAACAATTCCC-3'

These oligonucleotides were used for synthesis of the following DNA sequences:

A. Synthetic Shine-Dalgarno Sequence

The oligonucleotides A and B were kinased by incubating a mixture of 5 µg of the two oligonucleotides for 1 h at 37°C with 60 µl of 1mM ATP, 5 µl of 10 × kinase mix (0.5M Tris-HCl pH 7.0, 0.1M $MgCl_2$, 50mM dithiothreitol, 1 mM spermidine, 1mM EDTA) and 3 µl of T_4-kinase (Gibco, 10U/µl) in a volume of 50 µl. The kinased oligonucleotides were subsequently annealed by 5 min. incubation at 100°C followed by 30 min. incubation at 65°C. After purification with phenol-chloroform (1:1) and precipitation with ethanol, the DNA was resuspended in a small volume of TE buffer.

B. Synthetic promoter-sequence recognized by σ^{55}-RNA polymerase

Analogously to the description under A, but starting from a mixture of the oligonucleotides C, D, E and F, a synthetic promoter sequence recognized by σ^{55}-RNA polymerase was obtained.

C. Synthetic promoter-sequence recognized by σ^{37}-RNA polymerase

Analogously to the description under A, but starting from a mixture of the oligonucleotides G, H, I and J, a synthetic promoter sequence recognized by σ^{37}-RNA polymerase was obtained.

Example IV

Construction of a Shine-Dalgarno/signal sequence construct in plasmid pPROM 54

15 µg of pGB 33, isolated from *B. subtilis* 1—85 (see Example II) was cut with the restriction enzyme *Nde*I, of which the recognition site is located precisely between the promoter and the Shine-Dalgarno sequence of the *B. licheniformis* alpha-amylase gene, as may be seen from the sequence analysis (see Fig. 1). Aft r extraction with ph n l-chloroform (1:1) and precipitation with ethanol, th digested plasmid-DNA was resuspended in 59 µl of *Bal*31 mix (120 µl of 100mM Tris-HCl pH 8.1, 72 µl of 100mM $MgCl_2$, 72 µl of 100mM $CaCl_2$, 120 µl of 1M NaCl, 156 µl of H_2O and 1 µl of *Bal*31 exonuclease (Gibco 1.2U/µl)). After 3.4 min. incubation at 15°C, the material was again extracted with phen l-chloroform (1:1) and reprecipitated with ethanol. After resuspending, the DNA was digested with the restriction enzyme *Pst*I, extracted with phenol-chloroform (1:1), precipitated with ethanol and resuspended in 20 µl f a ligase mix which contained 20mM

Tris-HCl pH 7.6, 10mM $MgCl_2$, 10mM dithi threitol, 0.5mM ATP, 1 µl *E. coli* phage M123mp10 (digested with the restricti n enzymes *Hinc*II and *Pst*I) and 1 µl of T_4 ligase (B hringer 1U/µl), after which ligation was carried out overnight at 4°C (see fig. 2).

After transformation and selection of white plaques in *E. coli,* a number of recombinant DNA phages were isolated and sequenced with the aid of the "dideoxychain terminator" method. In the recombinant most shortened by *Bal*31 (see Fig. 2) the synthetic Shine-Dalgarno sequence (see Example III) was inserted after digestion with the restriction enzymes *Bam*HI and *Xba*I, after which the construct was sequenced. This fragment containing the Shine-Dalgarno sequence was subsequently excised with restriction enzymes *Eco*RI and *Pst*I (see Fig. 2) and substituted for the *Eco*RI-*Pst*I fragment carrying the original regulation signals of the *B. licheniformis* alpha-amylase gene. The plasmid thus obtained, pPROM 54, has a size of ± 5.2kbp. The structure of the plasmid is shown in Fig. 3.

The plasmid pPROM 54 in *B. subtilis* 1A40 (amy^-, lys^-, met^-, trp^-) was deposited at the CBS on November 5, 1985 under No. 696.85.

Example V

Construction of pPROM plasmids which comprise chromosomal promoter sequences

5 µg of chromosomal DNA, isolated from *B. licheniformis* strain T5 (Example I) were cut with *Rsa*I, *Hae*III, *Alu*I, *Hinc*II or *Eco*RV and, after purification with phenol-chloroform and precipitation with ethanol, were ligated to 1 µg of pPROM 54 (Example IV), restricted with *Sma*I. Another portion of chromosomal DNA from *B. licheniformis* strain T_5 was digested with *Eco*RI under Eco* conditions, purified, precipitated and ligated to 1 µg of pPROM 54, linearised with *Eco*RI. The ligated mixtures were transformed into *B. subtilis* 1A40 (amy^-, lys^-, met^-, trp^-) using the method described by *Anagnostopoulos and Spizizen,* J. Bacteriol. (1981) *81*:741—746. Transformants were first selected for neomycin/kanamycin resistance on minimal agar plates to which 1% (w/v) of starch, 0.02% (w/v) of casamino acids (Difco) and 10 µg/ml neomycin were added.

These transformants were subsequently analyzed for the presence of a promoter sequence by selection in respect of the capacity achieved for the synthesis of alpha-amylase, which was done by looking for halos after having poured a solution of 0.6% (w/v) of KI and 0.3% (w/v) of I_2 over the plates. The transformants thus selected were used for fermentative production of alpha-amylase in comparison with production under the influence of the native alpha-amylase regulatory region. The selected transformants were also used as the source for recombinant DNA plasmids.

One of the selected transformants comprised the plasmid pPROM 3—4C. This plasmid in *B. subtilis* 1A40 (amy^-, lys^-, met^-, trp^-), was deposited at the CBS on November 5, 1985 under No. 699.85.

Example VI

Construction of pPROM plasmids which comprise bacteriophage promoter sequences

5 µg of pPL 608 carrying an SPO_2 phage promoter fragment of 280bp *(Williams et al.,* J. Bacteriol. (1981) *146*:1162—1165), were cut with *Eco*RI and, after purification and precipitation, were ligated to 1 µg of pPROM 54, linearized with *Eco*RI. The ligated mixture was transformed into *B. subtilis* 1A40 (amy^-, lys^-, met^-, trp^-). The transformants obtained were selected in the manner described in Example V. The selected transformant comprised the plasmid pPROM SPO_2. This transformant was used for fermentative production of alpha-amylase in comparison with the production under the influence of the native alpha-amylase gene and also used as as source for the recombinant plasmid pPROM SPO_2.

The plasmid pPROM SPO_2 in *B. subtilis* 1A40 (amy^-, lys^-, met^-) was deposited at the CBS on 5th November 1985 under No. 698.85.

Example VII

Construction of pPROM plasmids which comprise synthetic promoter sequences

5 µg of the synthetic promoter obtained by purification and annealing of the oligonucleotides C, D, E and F (see Example III) were ligated to 1 µg of pPROM 54, digested with *Eco*RI and *Sst*I (see Fig. 4). The ligated mixture was transformed into *B. subtilis* 1A40 (amy^-, lys^-, met^-). The transformants obtained were selected in the manner described in Example V.

The selected transformant comprised the plasmid pPROM 55s. The transformant was used for fermentative production of alpha-amylase in comparison with the production under the influence of the native alpha-amylase gene, and also as a source for the recombinant plasmid pPROM 55s.

The plasmid pPROM 55s in *B. subtilis* 1A40 (amy^-, lys^-, met^-, trp^-) was deposited at the CBS on November 5, 1985 under No. 697.85.

Analogously to this procedure, but starting from the synthetic oligonucleotides G, H, I and J (see Example III), a *Bacillus* 1A40 transformant containing th recombinant plasmid pPROM 37S, was obtained, which plasmid differs from pPROM 55s in respect of the synthetic promoter sequence (compare Figs. 4 and 5).

Example VIII

Fermentative pr ducti n f alpha-amylase with the aid f genetically manipulated Bacillus subtilis strains

Th *B. subtilis* strains obtained after genetic manipulation as described in Exampl s V, VI and VII, and

also the *B. subtilis* strain with the starting plasmid pGB 33, were cultured for 5 days at 37°C in a liquid h art infusion medium made up with 0.4% of Zulkowski starch. The alpha-amylase was isolated and purified in accordance with standard procedures. The quantities of alpha-amylase produced, in comparison with the original *B. subtilis* strain having the starting plasmid pGB 33 (bearing the unmodified alpha-amylas gene) are shown in Table I.

Table I

Origin of promoter	Clone no.	α-amylase production (TAU/ml)	% compared with control
B.lich. chromosomal DNA x Rsa I	pPROM 1.1	48.2	116
x Rsa I	pPROM 2.6	44.3	107
x Alu I	pPROM 11.1	47.0	113
x Alu I	pPROM 14.3	43.4	117
x Hinc II	pPROM 17.4	39.7	96
x Hinc II	pPROM 17.5	36.4	88
x Hinc II	pPROM 17.6	43.5	105
x EcoR V	pPROM 23.25	45.5	110
x EcoR V	pPROM 23.26	49.3	119
x EcoR I*	pPROM 3-4C	55.8	135
pPL 608 DNA x EcoR I	pPROM SPO_2	56.8	137
Synthetic DNA	pPROM 37S	39.3	95
	pPROM 55S	33.1	80
Control	pGB 33	41.4	100

In accordance with the subject invention, functional sequences can be readily isolated and evaluated by substitution or insertion of regulatory regions, signal sequences, or structural genes into a designed construct. The resulting constructs may then be introduced into a *Bacillus* host and the efficiency of expression and secretion determined. In this manner, *Bacillus* libraries or libraries from other hosts which may have regulatory regions functional in *Bacillus* may be screened for their use in *Bacillus*. Thus, promoters, ribosomal binding sites and signal sequences may be evaluated from a wide variety of hosts, such as viruses, microorganisms, and the like.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that changes and modifications may be practised within the scope of the appended claims.

Claims

1. A plasmid capable of replication in *Bacillus* useful in evaluating regulat ry or signal sequ nces for expression of a hybrid gene having a domain comprising at least one restriction site and as elements f r substituti n: (1) as a pr moter region, the *B. licheniformis* prom t r s quence ion pPROM 3—4C, which plasmid has the deposit accession number CBS 699.85, the bacteriophage promoter of pPROM SPO2,

which plasmid has the dep sit accession number CBS 698.85, or th −10 and −35 sequences of a synthetic promoter region, (2) a ribosomal binding site, with the pr viso that when said pr moter region is absent, said ribos mal binding site is synthetic, and (3) a signal sequence functional in *Bacillus;* with unique restriction sites between the −10 and −35 consensus sequences of any synthetic promoter region, between the promoter region and the ribosomal binding site, proximal to the initiation codon and at the 3'-terminus of the signal sequence, wherein at least two of said elements are not naturally linked, with the proviso that one of said elements may be replaced by a unique restriction site, and in that event said signal sequence is the alpha-amylase signal sequence and is joined to a structural gene in reading phase encoding mature alpha-amylase.

2. A plasmid according to Claim 1, wherein said domain comprises at least one of the following sequences:

A) 5'-GATCCAAGGAGGTGAT-3'

B) 5'-CTAGATCACCTCCTTG-3'

C) 5'-AATTCTTGACAAAGCTTC-3'

D) 5'-TCGAGAAGCTTTGTCAAG-3'

E) 5'-TCGAGACTGATATAATGAGCT-3'

F) 5'-CATTATATCAGTC-3'

G) 5'-AATTCAGGATTTATGAAGCTTC-3'

H) 5'-TCGAGAAGCTTCATAAATCCTG-3'

I) 5'-TCGAGGGAATTGTTTGAGCT-3'

J) 5'-CAAACAATTCCC-3'

3. A plasmid according to Claim 1, wherein said promoter is replaced with a sequence which includes at least three unique restriction sites.

4. A plasmid according to Claim 1, wherein said promoter specifically binds to the σ^{55}-RNA polymerase.

5. A plasmid according to Claim 4, wherein said promoter has the sequence:

AATTCTTGACAAAGCTTCTCGAGACTGATATAATGAGCT

GAACTGTTTCGAAGAGCTCTGACTATATTAC

6. A plasmid according to Claim 1, wherein said promoter specifically binds to the σ^{37}-RNA polymerase.

7. A plasmid according to Claim 6, wherein said promoter has the sequence:

AATTCAGGATTTATGAAGCTTCTCGAGGGAATTGTTTGAGCT

GTCCTAAATACTTCGAAGAGCTCCCTTAACAAAC

8. A plasmid according to Claim 1, wherein said plasmid comprises the replication system of pUB110.

9. A plasmid according to Claim 1, wherein said plasmid comprises at least one gene imparting antibiotic resistance to *Bacillus.*

10. A plasmid according to Claim 1, wherein said ribosomal binding site has th sequence:

GATCCAAGGAGGTGAT

GTTCCTCCACTAGATC

11. A plasmid according t Claim 1, consisting essentially of pPROM 54, pPROM 55s, pPROM SP02 or pPROM 3—4C, said plasmids having the deposit accession numb rs CBS 696.85—CBS 699.85, resp ctively.

12. A plasmid acc rding to Claim 11, wherein at least one but not all of the elements are substituted by a different sequence having the same function.

13. A plasmid according to Claim 1, comprising in reading phase with said signal sequence a structural gene joined to said sequence.

14. A *Bacillus* host comprising a plasmid according to any of Claims 1 to 13.

15. A *Bacillus subtilis* host comprising a plasmid according to any of Claims 1 to 13.

16. A method for determining the efficiency of one or more functional elements in the production of a peptide in a *Bacillus* host, said method comprising:

growing said host in an appropriate nutrient medium, said host comprising a plasmid capable of replication in *Bacillus* having a domain comprising as elements for substitution: (1) as a promoter region, the *B. licheniformis* promoter sequence in pPROM 3—4C, which plasmid has the deposit accession number CBS 699.85, the bacteriophage promoter of pPROM SP02, which plasmid has the deposit accession number CBS 698.85, or the −10 and −35 sequences of a synthetic promoter region; (2) a ribosomal binding site, and (3) a signal sequence functional in *Bacillus*; with unique restriction sites between the −10 and −35 consensus sequences of the synthetic promoter region, between the promoter region and the ribosomal binding site, proximal to the initiation codon and at the 3'-terminus of the signal sequence, wherein at least one but not all of said elements are substituted by said functional element(s) and wherein a structural gene is in reading phase with said signal sequence; and

determining the amount of said peptide produced in comparison with the amount of peptide produced under comparable conditions with said domain prior to any substitution with said functional elements.

17. A method for detecting the presence of a sequence capable of initiating transcription in *Bacillus,* said method comprising:

fragmenting DNA from a host having transcriptional initiation regions to less than about 5kbp to produce DNA fragments;

inserting said DNA fragments into a plasmid capable of replication in *Bacillus* and having means for selection of transformants at a restriction site region having at least one unique restriction site and being the upstream region of a screening region, said screening region comprising in the direction of transcription, said restriction site region, a ribosomal binding site region, and a structural gene having a signal sequence and capable of secretion in *Bacillus* to produce a product which is readily detectable by a chemical reaction;

transforming *Bacillus* cells with said plasmid to produce transformants and selecting for transformants by means of said selection means;

screening transformants for secretion of said product by means of said chemical reaction to determine the presence of a fragment at said restriction site having transcriptional initiation capability.

18. A method according to Claim 17, wherein said ribosomal binding site region is a synthetic region and said structural gene encodes alpha-amylase.

Patentansprüche

1. Plasmid, das zur Replikation in Bacillus befähigt und für die Evaluation von regulatorischen oder Signalsequenzen für die Expression eines Hybridgens brauchbar ist, welches Hybridgen eine Domäne hat, die mindestens eine Restriktionsstelle aufweist und als Elemente für den Austausch aufweist:

(1) als Promotorregion die B. licheniformis-Promotorsequenz in pPROM 3—4C, welches Plasmid die Hinterlegungs-Ordnungsnummer CBS 699.85 hat, den Bakteriophagen-Promotor von pPROM SP02, welches Plasmid die Hinterlegungs-Ordnungsnumme CBS 698.85 hat, oder die −10-und −35-Sequenzen einer synthetischen Promotorregion,

(2) eine Ribosomen-Bindungsstelle, vorausgesetzt, dass die Ribosomen-Bindungsstelle synthetisch ist, wenn die genannte Promotorregion fehlt, und

(3) eine Signalsequenz, die in Bacillus funktionsfähig ist; mit singulären Restriktionsstellen zwischen den −10 und −35-Consensussequenzen einer allfälligen synthetischen Promotorregion, zwischen der Promotorregion und der Ribosomen-Bindungsstelle, proximal in bezug auf das Initiationscodon und an dem 3'-Ende der Signalsequenz, worin mindestens zwei der genannten Elemente nicht natürlich verknüpft sind, vorausgesetzt, dass eines der genannten Elemente durch eine singuläre Restriktionsstelle ersetzt sein kann und die genannten Signalsequenz in diesem Falle die alpha-Amylase-Signalsequenz ist und in Lesephase mit einem Strukturgen verbunden ist, das für aktive alpha-Amylase codiert.

2. Plasmid nach Anspruch 1, worin die genannte Domäne mindestens eine der folgenden Sequenzen aufweist:

A) 5'-GATCCAAGGAGGTGAT-3'

B) 5'-CTAGATCACCTCCTTG-3'

C) 5'-AATTCTTGACAAAGCTTC-3'

D) 5'-TCGAGAAGCTTTGTCAAG-3'

E) 5'-TCGAGACTGATATAATGAGCT-3'

F) 5'-CATTATATCAGTC-3'

G) 5'-AATTCAGGATTTATGAAGCTTC-3'

H) 5'-TCGAGAAGCTTCATAAATCCTG-3'

I) 5'-TCGAGGGAATTGTTTGAGCT-3'

J) 5'-CAAACAATTCCC-3'

3. Plasmid nach Anspruch 1, worin der genannte Promotor durch eine Sequenz ersetzt ist, die mindestens drei singuläre Restriktionsstellen einschliesst.

4. Plasmid nach Anspruch 1, worin der genannte Promotor spezifisch an die sigma^{55}-RNA-Polymerase bindet.

5. Plasmid nach Anspruch 4, worin der genannte Promotor die Sequenz:

AATTCTTGACAAAGCTTCTCGAGACTGATATAATGAGCT

GAACTGTTTCGAAGAGCTCTGACTATATTAC

hat.

6. Plasmid nach Anspruch 1, worin der genannte Promotor spezifisch an die sigma^{37}-RNA-Polymerase bindet.

7. Plasmid nach Anspruch 6, worin der genannte Promotor die Sequenz:

AATTCAGGATTTATGAAGCTTCTCGAGGGAATTGTTTGAGCT

GTCCTAAATACTTCGAAGAGCTCCCTTAACAAAC

hat.

8. Plasmid nach Anspruch 1, worin das genannte Plasmid das Replikationssystem von pUB110 aufweist.

9. Plasmid nach Anspruch 1, worin das genannte Plasmid mindestens ein Gen aufweist, das Bacillus Antibiotikaresistenz verleiht.

10. Plasmid nach Anspruch 1, worin die genannte Ribosomen-Bindungsstelle die Sequenz:

GATCCAAGGAGGTGAT

GTTCCTCCACTAGATC

hat.

11. Plasmid nach Anspruch 1, das im wesentlichen aus pPROM 54, pPROM 55s, pPROM SPO2 oder pPROM 3—4C besteht, wobei die genannten Plasmide die Hinterlegungs-Ordnungsnummern CBS 696.85 bis CBS 699.85 haben.

12. Plasmid nach Anspruch 11, worin mindestens eines, aber nicht alle der Elemente durch eine andere Sequenz mit der gleichen Funktion ersetzt sind.

13. Plasmid nach Anspruch 1, das in Lesephase mit d r genannten Signalsequenz ein Strukturgen aufweist, das mit der genannten Sequenz verbunden ist.

14. Bacillus-Wirt, der ein Plasmid nach einem der Ansprüche 1 bis 13 aufweist.

15. Bacillus subtilis-Wirt, der ein Plasmid nach einem der Ansprüche 1 bis 13 aufweist.

16. Verfahr n zur Bestimmung der Effizienz eines oder mehrerer funktioneller Elemente bei der

Produktion eines Peptids in einem Bacillus-Wirt, welches Verfahren umfasst:

das Kultivieren des Wirtes in einem geeigneten Nährmedium, wobei der Wirt ein Plasmid aufweist, das zur Replikation in Bacillus b fähigt ist und eine Domäne hat, die als El mente für den Austausch aufw ist:

(1) als Pr mot rregion die B. licheniformis-Pr mot rsequenz in pPROM 3—4C, welches Plasmid die Hinterlegungs-Ordnungsnummer CBS 699.85 hat, den Bakteriophagen-Promotor von pPROM SP02, welches Plasmid die Hinterlegungs-Ordnungsnummer CBS 698.85 hat, oder die −10- und −35-Sequenzen einer synthetischen Promotorregion,

(2) eine Ribosomen-Bindungsstelle und

(3) eine Signalsequenz, die in Bacillus funktionsfähig ist; mit singulären Restriktionsstellen zwischen den −10 und −35-Consensussequenzen der synthetischen Promotorregion, zwischen der Promotorregion und der Ribosomen-Bindungsstelle, proximal in bezug auf das Initiationscodon und an dem 3'-Ende der Signalsequenz, worin mindestens eines, aber nicht alle der genannten Elemente durch das genannte funktionelle Element oder die genannten funktionellen Elemente ersetzt ist bzw. sind und worin ein Strukturgen in Lesephase mit der genannten Signalsequenz ist; und

das Bestimmen der erzeugten Menge des genannten Peptids im Vergleich mit der Menge an Peptid, die unter vergleichbaren Bedingungen mit der genannten Domäne vor irgendwelchem Ersatz durch die genannten funktionellen Elemente erzeugt wird.

17. Verfahren zum Nachweis des Vorhandenseins einer Sequenz, die zur Initiation der Transkription in Bacillus befähigt ist, welches Verfahren umfasst:

das Fragmentieren von DNA aus einem Wirt mit Initiationsregionen für die Transkription kleiner als ca. 5 kbp zur Erzeugung von DNA-Fragmenten;

das Inserieren der genannten DNA-Fragmente in ein Plasmid, das zur Replikation in Bacillus befähigt ist und Mittel zur Selektion von Transformanten an einer Restriktionsstellenregion hat, die mindestens eine singuläre Restriktionsstelle hat und die weiter oben liegende Region einer Screening-Region ist, welche letztere Region in Transkriptionsrichtung die genannte Restriktionsstellenregion, eine Ribosomen-Bindungsstellenregion und ein Strukturgen aufweist, welch letzteres eine Signalsequenz hat und zur Sekretion in Bacillus befähigt ist, um ein Produkt zu erzeugen, das durch eine chemische Reaktion leicht nachweisbar ist;

das Transformieren von Bacillus-Zellen mit dem genannten Plasmid, um Transformanten zu erzeugen, und das Selektionieren auf Transformanten mit Hilfe der genannten Selektionsmittel;

das Testen der Transformanten auf die Sekretion des genannten Produktes mit Hilfe der genannten chemischen Reaktion, um das Vorhandensein eines Fragmentes mit der Fähigkeit zur Initiation der Transformation an der genannten Restriktionsstelle zu ermitteln.

18. Verfahren nach Anspruch 17, worin die genannte Ribosomen-Bindungsstellenregion eine synthetische Region ist und das genannte Strukturgen für alpha-Amylase codiert.

Revendications

1. Plasmide capable de réplication dans *Bacillus*, utile pour l'évaluation des séquences régulatrices ou signal pour l'expression d'un gène hybride, ayant un domaine comprenant au moins un site de restriction et comme éléments pour la substitution: (1) comme région promoteur, la séquence promoteur de *B. licheniformis* dans pPROM 3-4C, lequel plasmide a le numéro d'accès au dépôt CBS 699.85, le promoteur bactériophage de pPROM SP02, lequel plasmide a le numéro d'accès au dépôt CBS 698.85, ou les séquences −10 et −35 d'une région promoteur synthétique, (2) un site de fixation ribosomique, avec la restriction que lorsque la région promoteur est absente, le site de fixation ribosomique est synthétique, et (3) une séquence signal fonctionnelle dans *Bacillus*; avec des sites de restriction uniques entre les séquences consensus −10 et −35 de toute région promoteur synthétique, entre la région promoteur et le site de fixation ribosomique, proximal du codon d'initiation et à l'extrémité 3' de la séquence signal, dans lequel au moins deux de ces éléments ne sont pas naturellement liés, avec la restriction que l'un de ces éléments peut être remplacé par un site de restriction unique, et dans ce cas, cette séquence signal est la séquence signal de l'alpha-amylase et est unie à un gène de structure en phase de lecture codant pour l'alpha-amylase mature.

2. Plasmide suivant la revendication 1, dans lequel le domaine comprend au moins l'une des séquences suivantes:

A) 5'-GATCCAAGGAGGTGAT-3'

B) 5'-CTAGATCACCTCCTTG-3'

C) 5'-AATTCTTGACAAAGCTTC-3'

D) 5'-TCGAGAAGCTTTGTCAAG-3'

E) 5'-TCGAGACTGATATAATGAGCT-3'

F) 5'-CATTATATCAGTC-3'

G) 5'-AATTCAGGATTTATGAAGCTTC-3'

H) 5'-TCGAGAAGCTTCATAAATCCTG-3'

I) 5'-TCGAGGGAATTGTTTGAGCT-3'

J) 5'-CAAACAATTCCC-3'

3. Plasmide suivant la revendication 1, dans lequel le promoteur est remplacé par une séquence qui inclut au moins trois sites de restriction uniques.

4. Plasmide suivant la revendication 1, dans lequel le promoteur se fixe spécifiquement sur l'ARN polymérase de σ^{55}.

5. Plasmide suivant la revendication 4, dans lequel le promoteur a la séquence:

AATTCTTGACAAAGCTTCTCGAGACTGATATAATGAGCT

GAACTGTTTCGAAGAGCTCTGACTATATTAC

6. Plasmide suivant la revendication 1, dans lequel le promoteur se fixe spécifiquement sur l'ARN polymérase de σ^{37}.

7. Plasmide suivant la revendication 6, dans lequel le promoteur a la séquence:

AATTCAGGATTTATGAAGCTTCTCGAGGGAATTGTTTGAGCT

GTCCTAAATACTTCGAAGAGCTCCCTTAACAAAC

8. Plasmide suivant la revendication 1, dans lequel le plasmide comprend le système de réplication de pUB110.

9. Plasmide suivant la revendication 1, dans lequel le plasmide comprend au moins un gène conférant à *Bacillus* la résistance à un antibiotique.

10. Plasmide suivant la revendication 1, dans lequel le site de fixation ribosomique a la séquence:

GATCCAAGGAGGTGAT

GTTCCTCCACTAGATC

11. Plasmide suivant la revendication 1, consistant essentiellement en pPROM 54, pPROM 55s, pPROM SP02 ou pPROM 3—4C, lesquels plasmides ont les numéros d'accès au dépôt CBS 696.85 — CBS 699.85, respectivement.

12. Plasmide suivant la revendication 11, dans lequel au moins un des éléments mais non tous est substitué par une séquence différente ayant la même fonction.

13. Plasmide suivant la revendication 1, comprenant en phase de lecture avec la séquence signal un gène de structure uni à la séquence.

14. *Bacillus* hôte comprenant un plasmide suivant l'une quelconque des revendications 1 à 13.

15. *Bacillus subtilis* hôte comprenant un plasmide suivant l'une quelconque des revendications 1 à 13.

16. Procédé pour déterminer l'efficacité d'un ou plusieurs éléments fonctionnels dans la production d'un peptide dans un *Bacillus* hôte, lequel procédé comprend:

la culture de l'hôte dans un milieu nutritif approprié, l'hôte comprenant un plasmide capabl de réplication dans *Bacillus*, ayant un domaine comprenant comme éléments pour la substitution: (1) comme régi n promoteur, la séquence promoteur de *B. licheniformis* dans pPROM 3—4C, lequel plasmide a l numér d'accès au dépôt CBS 699.85, le promoteur bactéri phag de pPROM SP02, lequel plasmid a l numéro d'accès au dépôt 698.85, ou bien les séquences −10 et −35 d'une région promoteur synthétique; (2) un site de fixation ribosomique, et (3) une séquence signal fonctionnelle dans *Bacillus*; avec des sites de restriction uniques entre les séquences consensus −10 et −35 de la région promoteur synthétique, entre la

r´gion promoteur et le site de fixation ribos mique, proximal du cod n d'initiation et à l'extrémité 3' de la séquence signal, dans lequel au moins un de ces éléments mais non t us est substitué par le ou les éléments fonctionnels et dans lequel un gène de structure est en phase de lecture avec la séquence signal; et

la détermination de la quantité du peptide produit par comparaison avec la quantité du peptide produit dans des conditions comparables avec ce domaine avant toute substitution à l'aide de ces éléments fonctionnels.

17. Procédé pour détecter la présence d'une séquence capable d'initier la transcription dans *Bacillus,* lequel procédé comprend:

la fragmentation de l'ADN d'un hôte ayant des régions d'initiation de transcription jusqu'à moins d'environ 5kbp pour produire des fragments d'ADN;

l'insertion des ces fragments d'ADN dans un plasmide capable de réplication dans *Bacillus* et ayant des moyens pour la sélection de transformants à une région de site de restriction ayant au moins un site de restriction unique et étant la région à l'amont d'une région de débrouillement, la région de débrouillement comprenant dans la direction de transcription, la région du site de restriction, une région de site de fixation ribosomique et un gène de structure ayant la séquence signal et capable de sécrétion dans *Bacillus* pour produire un produit quie est aisément détectable par une réaction chimique;

la transformation de cellules de *Bacillus* au moyen de ce plasmide pour produire des transformants et la sélection des transformants à l'aide de ces moyens de sélection;

le débrouillement des transformants en fonction de la sécrétion du produit à l'aide de la réaction chimique pour déterminer la présence d'un fragment à ce site de restriction ayant une capacité d'initiation de transcription.

18. Procédé suivant la revendication 17, dans lequel la région du site de fixation ribosomique est une région synthétique et le gène de structure code pour l'alpha-amylase.

FIG. 1

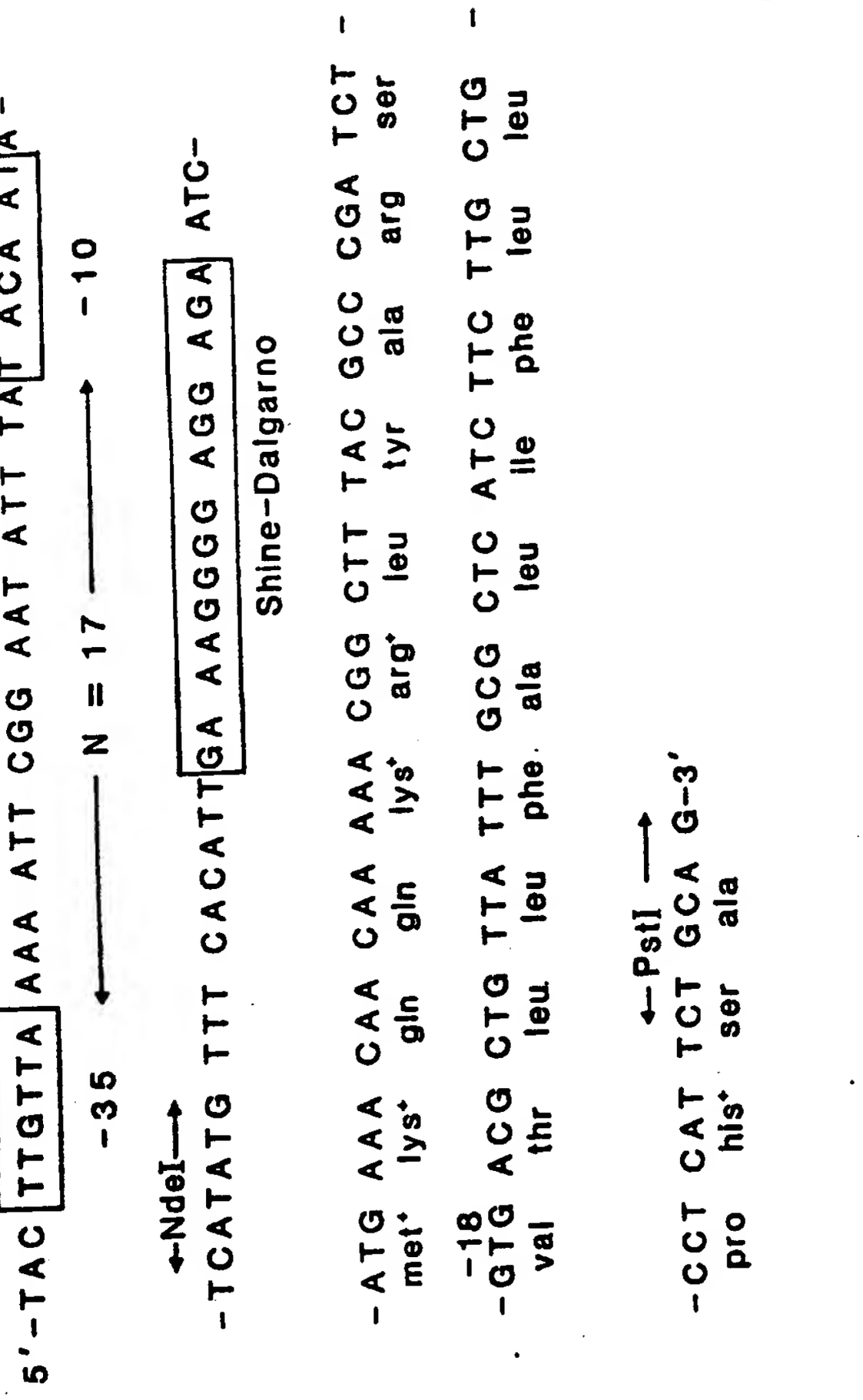

5'-TAC TTGTTA AAA ATT CGG AAT ATT TA T ACA AT A-
-35
N = 17
-10
←NdeI→
-TCATATG TTT CACATT GA AAGGGG AGG AGA ATC-
Shine-Dalgarno
-ATG AAA CAA AAA CGG CTT TAC GCC CGA TC
met' lys' gln lys' arg' leu tyr ala arg se
-18
-GTG ACG CTG TTA TTT GCG CTC ATC TTC TTG CT
val thr leu leu phe ala leu ile phe leu le
←PstI
-CCT CAT TCT GCA G-3'
pro his' ser ala

FIG. 2

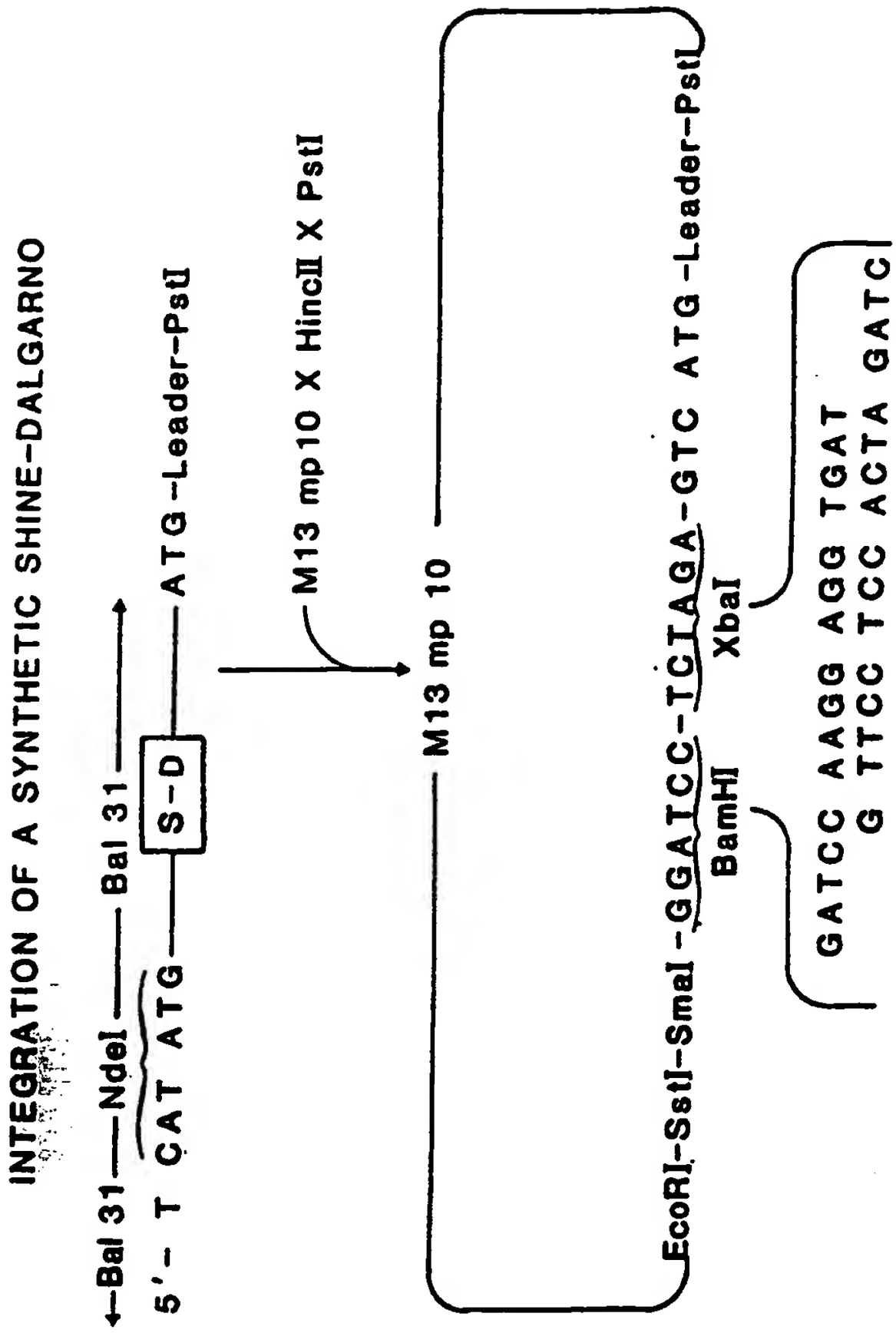

INTEGRATION OF A SYNTHETIC SHINE-DALGARNO
←Bal 31—NdeI—ATG—S-D—ATG-Leader-PstI
5'- T CAT ATG
M13 mp10 X HincII X PstI
M13 mp 10
EcoRI-SstI-SmaI-GGATCC-TCTAGA-GTC ATG-Leader-PstI
BamHI XbaI
GATCC AAGG AGG TGAT
G TTCC TCC ACTA GATC
Synth. Shine-Dalgarno
N = 12 bp ΔG = -28.4 Kcal. mol-1

FIG. 3

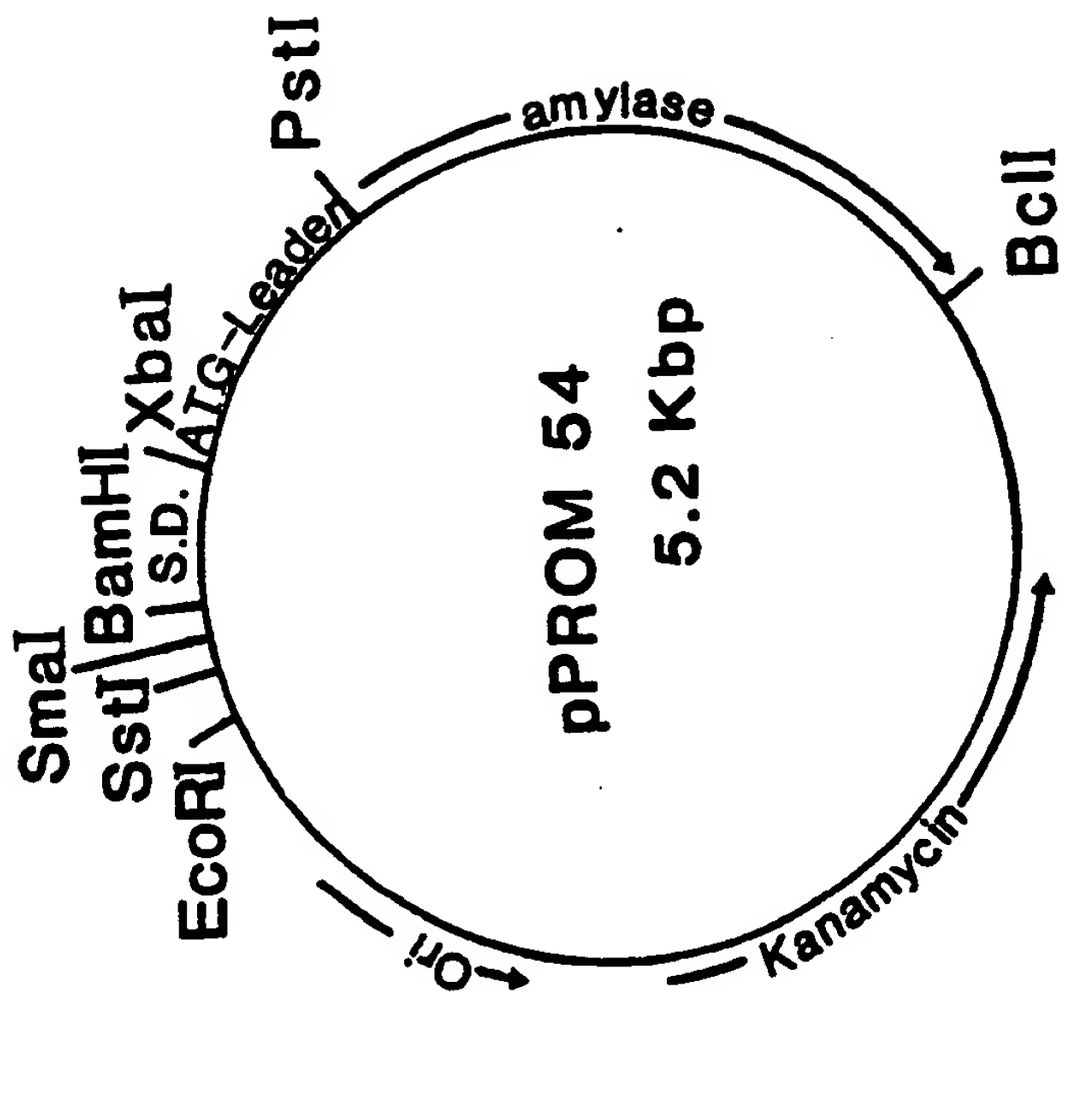

pPROM 54
5.2 Kbp
amylase
PstI
BclI
Kanamycin
Ori
SmaI
SstI
BamHI
XbaI
EcoRI
S.D.
ATG-Leader

FIG. 4

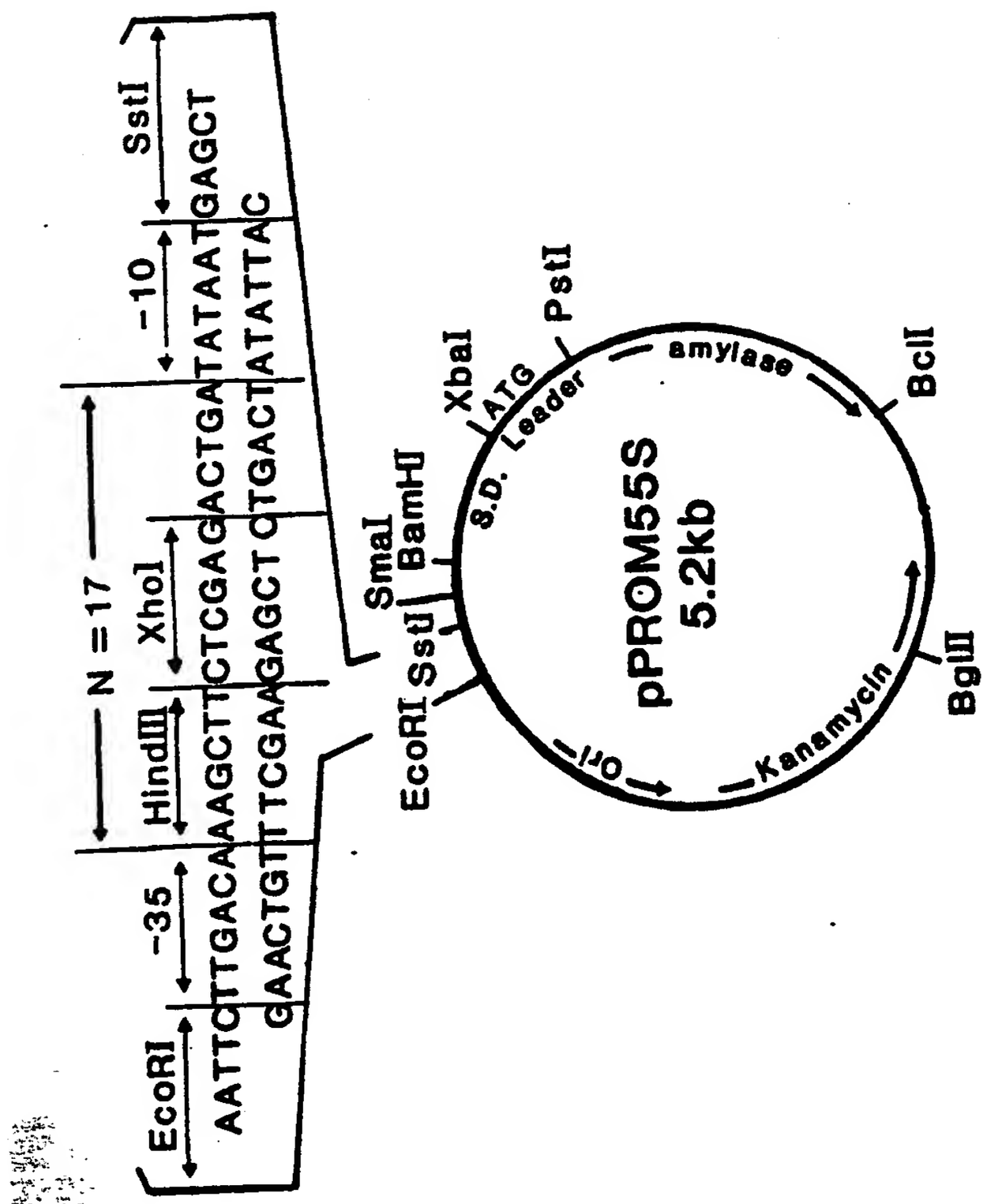
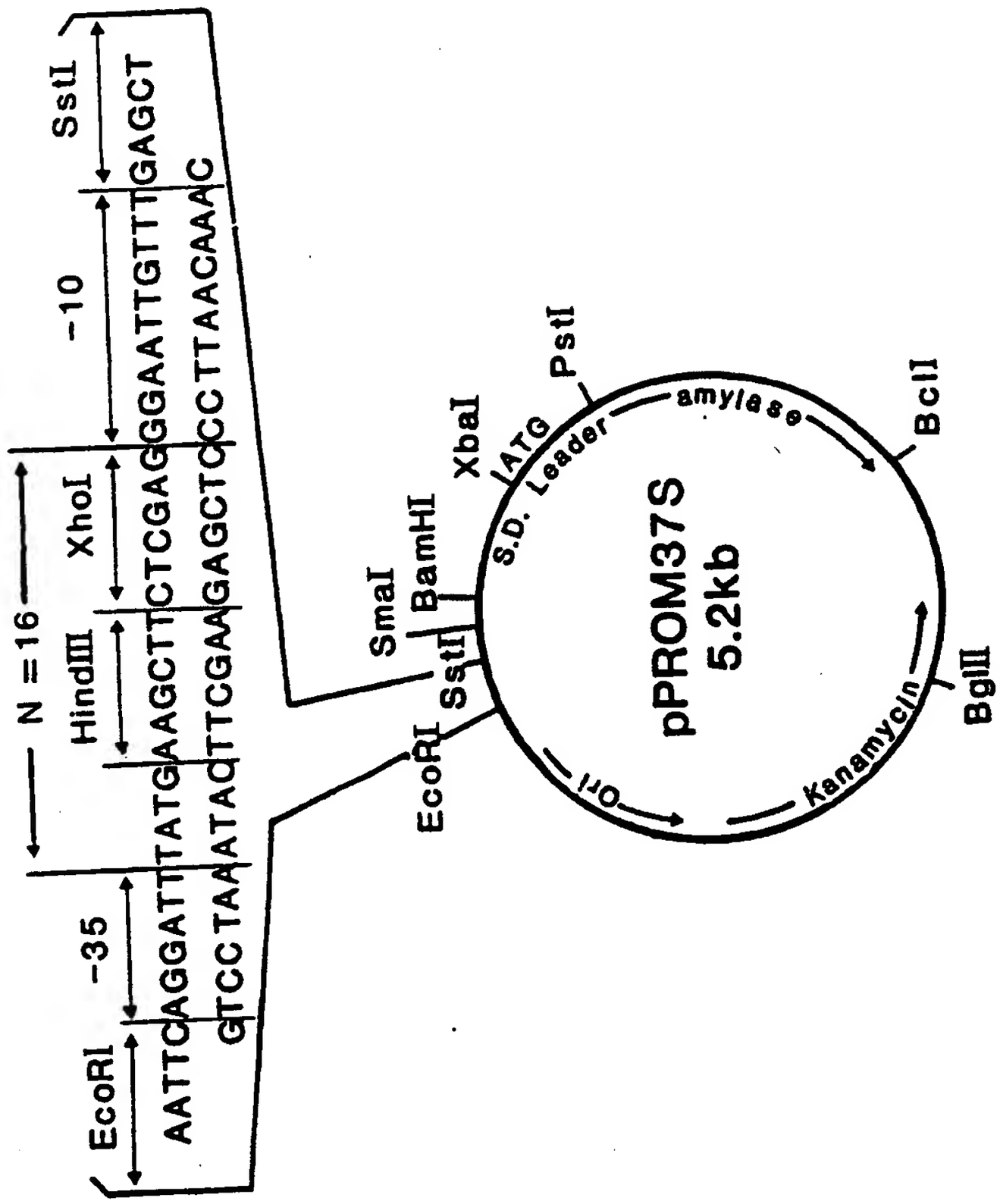

EcoRI
AATTC
G
SstI
-35
TTGACA
AACTGT
N = 17
HindIII
AAGCTT
TCGAA
XhoI
CTCGAG
GAGCT
SmaI
BamHI
SstI
EcoRI
-10
TATAAT
TATTAC
GACTGA
CTGACT
SstI
GAGCT
XbaI
S.D.
ATG
Leader
PstI
amylase
BclI
pPROM55S
5.2kb
ori
Kanamycin
BglII

FIG. 5


EcoRI
-35
AATTCAGGATTTATGA
GTCCTAAATACT
N = 16
HindIII
XhoI
-10
AAGCTTCTCGAGGGAATTGT
TTCGAAGAGCTCCCTTAACA
SmaI
BamHI
XbaI
PstI
SstI
EcoRI
S.D.
ATG
Leader
amylase
BclI
pPROM37S
5.2kb
Ori
Kanamycin
BglII